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ENERGETICS OF CONTRACTILE ACTIVITY IN ISOLATED RADULA PROTRACTOR MUSCLES OF THE WHELK *BUSYCON CONTRARIUM*: ANAEROBIC END PRODUCT ACCUMULATION AND RELEASE

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ABSTRACT

Anaerobic energy metabolism during contractile activity was investigated in the isolated radula protractor muscle of the whelk *Busycon contrarium*. Spectrophotometric assay of enzyme activities in crude tissue extracts revealed particularly high pyruvate reductase activities with octopine dehydrogenase displaying the highest activity. During electrically induced isotonic contractions of the radula protractor muscles, the following end products, listed in order of increasing level, accumulated in the tissue: strombine, octopine and alanopine (the "opines"), and D-lactate. Pyruvate levels increased three-fold during muscle contraction, suggesting that pyruvate plays a key role in the regulation of the pyruvate reductases. The muscle released lactate, but none of the opines, into the incubation medium, with rates exceeding 3 μmoles $\cdot$ $min^{-1} \cdot g$ wet wt^{-1}. During the later phases of contraction, more D-lactate was released into the medium than accumulated in the muscle. We conclude that transport of D-lactate permits sustained flux through lactate dehydrogenase because of a reduction in product inhibition. Furthermore, we hypothesize that D-lactate transport may be coupled to H^+ export or OH^- import, which would then serve to regulate the extent of accumulation of glycolytically produced protons.

INTRODUCTION

The muscles of marine molluscs possess two distinctly different mechanisms of energy production during periods of reduced oxygen availabiliy. During environmental anaerobiosis—*i.e.,* whole-organism exposure to anoxia—aspartate and glycogen are cofermented, yielding succinate and alanine as end products (Gäde, 1983). The succinate pathway typically occurs at relatively low rates and is associated with a reverse Pasteur effect in these muscles (Storey, 1985). A number of molluscs may undergo functional anaerobiosis, where oxygen demand exceeds delivery. In this instance, only certain tissues are rendered anoxic (Gäde, 1983). Under these conditions, the glycolytic flux is several orders of magnitude higher than under conditions of environmental anaerobiosis (Livingstone, 1982; Gäde, 1983). The higher energy outputs necessary for burst activity are provided by glycogen fermentation and the shunting of pyruvate through pyruvate reductases such as lactate and opine dehydrogenases (Gäde and Grieshaber, 1986), resulting in the accumulation of D-lactate, octopine, alanopine, or strombine.

Opine dehydrogenases catalyze the reductive condensation of pyruvate and an amino acid according to the following general reactions:

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arginine + pyruvate + NADH ⇌ octopine + NAD
(ODH, octopine dehydrogenase)

alanine + pyruvate + NADH ⇌ alanopine + NAD
(ADH, alanopine dehydrogenase)

glycine + pyruvate + NADH ⇌ strombine + NAD
(SDH, strombine dehydrogenase)

Many molluscan muscles have the enzymatic potential for producing several opine end products as well as D-lactate since high activities of opine and D-lactate dehydrogenases may occur in the same tissue (Zammit and Newsholme, 1976; Livingstone *et al.,* 1983). For example, the pedal retractor (Baldwin *et al.,* 1981; Baldwin and England, 1982) and radula (Ellington, 1982) muscles of gastropods contain significant activities of all opine dehydrogenases as well as D-lactate dehydrogenase. The relative contribution of these enzymes to the maintenance of glycolytic flux during contractile activity, not yet fully explored, is considered in this paper.

The metabolic disposition of end products of anaerobic metabolism in molluscs (Ellington, 1983b) is poorly known. Propionate is released into the hemolymph of *Mytilus edulis* (Zurburg *et al.,* 1982), whereas succinate appears to accumulate in the hemolymph of the clam *Mercenaria mercenaria* (Korycan and Storey, 1983). Octopine is not released into the hemolymph during contractile activity in the giant scallop, *Placopecten magellicanus* (de Zwaan *et al.,* 1980). However, hemolymph octopine levels are slightly elevated after contractile activity or hypoxia in the cephalopods *Sepia officinalis* and *Loligo vulgaris* (Storey and Storey, 1979; Gäde, 1980). Alanopine and strombine appear not to be released from molluscan muscles. Octopine, alanopine, and strombine levels fall during recovery, indicating oxidation *in situ* (Ellington, 1983b).

The present study focuses on the metabolism of the radula protractor muscle of the large marine gastropod *Busycon contrarium.* This muscle possesses relatively high activities of lactate and opine dehydrogenases. The presence of several pyruvate reductases in the radula protractor muscle poses questions about the control of these enzymes and the disposition of their products. We show that electrical stimulation of this muscle while it is subjected to anoxia induces the formation of all opines as well as D-lactate. Interestingly, formation of D-lactate is much greater than that of the other end products, even though the activities of the opine dehydrogenases are much higher. Further, D-lactate is released from the exercising muscle into the medium while the opines are retained. End-product removal may enhance the formation of additional D-lactate and, as a result, large amounts of carbon can be shunted through lactate dehydrogenase allowing for higher, sustained glycolytic fluxes during anoxia.

Materials and Methods

Animals

Specimens of the whelk *Busycon contrarium* were collected off Alligator Point in Franklin County, Florida, and were maintained in the flowing-seawater system at the Florida State University Marine Laboratory near St. Theresa. Individuals used in experiments were transferred to the Florida State University campus, where they were maintained for brief periods in a recirculating seawater system.

Biochemicals

Biochemicals were purchased from Boehringer-Mannheim (Indianapolis) and Sigma Chemical Company (St. Louis). D-Lactate dehydrogenase, used to determine D-lactate, was purified from the muscle of the horseshoe crab *Limulus polyphemus*. Octopine dehydrogenase, used to assay for octopine, arginine, and arginine phosphate, was purified from the adductor muscles of the scallop *Argopecten irradians concentricus*. Succinyl Co A synthase, used in succinate assays, was a gift from Dr. William Bridger, Department of Biochemistry, University of Alberta, Edmonton.

Experimental procedure

Intact radula protractor muscles, dissected from the proboscis apparatus, were ligated at both ends with surgical silk and placed in a 5 × 75-mm muscle bath filled with 1.5 ml of MBL (Marine Biological Laboratory) formula artificial seawater buffered with 5 m*M* hydroxyethylpiperazine ethanesulfonic acid (pH = 7.8). One end of the muscle was fastened to a hook electrode and pulled into a rubber sleeve at the bottom of the muscle bath. The other end was attached to a Narco Biosystems isotonic myograph transducer with the silk suture. Muscles were suspended in the bath at 1.5 times their resting length (measured upon excision). Temperature was maintained by immersion of the bath in a larger water-filled vessel which was jacketed and controlled by a Brinkmann model RM 6 recirculating water bath (20°C). Contractions were recorded with the isotonic transducer connected to a Narco Biosystems model MK IV physiograph. A second electrode, inserted in the bath, delivered square-wave pulses (60 volts, 40 ms) at 2.5-s intervals from a Grass model SD9 stimulator. The bath was gassed with normocapnic nitrogen (0.05% CO_2) through a 75-mm, 22-gauge Luer lock syringe needle.

Each muscle preparation was fastened in the bath and bubbled with nitrogen for 15 min. Control experiments were terminated at this point, and the tissues were removed, blotted, and frozen in liquid nitrogen. Experimental groups were gassed as the controls were, and then subjected to various periods of electrical stimulation (2.5, 5, 10, and 15 min) in the presence of normocapnic nitrogen before being frozen. In all cases, the medium was decanted from the bath and stored at −70°C for later analysis.

Enzyme assays

Freshly dissected muscles were homogenized in 24 volumes of extraction buffer (50 m*M* triethanolamine, pH 7.4, 1 m*M* EDTA, 20 m*M* mercaptoethanol, 20% glycerol) with a Tekmar UltraTurrax tissue homogenizer. The homogenate was centrifuged at 12,000 × *g* for 20 min at 4°C. The supernatant was passed through a Sephadex G-25 column (1.5 × 14 cm) equilibrated with extraction buffer less glycerol, which removed low-molecular-weight compounds. The proteins in the void volume were used as the source of enzyme activities. The activities were determined spectrophotometrically with a Gilford model 252-1 spectrophotometer according to the methods outlined by Ellington (1982).

Metabolite assays

Nuetralized perchloric acid extracts were prepared from the radula protractor muscles frozen at −70°C according to the methods of Graham and Ellington (1985). Arginine phosphate was assayed spectrophotometrically by the method of Grieshaber

TABLE I

Profile of pyruvate reductase activities in desalted tissue extracts of radula protractor muscles from Busycon contrarium

Enzyme	Activity
D-Lactate dehydrogenase	36.46 ± 9.71
Strombine dehydrogenase	61.98 ± 14.88
Alanopine dehydrogenase	96.87 ± 19.66
Octopine dehydrogenase	509.83 ± 40.73

Activities are expressed in μmoles · min^{-1} g wet wt^{-1} and were measured at 25°C. Data represents mean ± 1 SD, n = 4.

and Gäde (1976). Pyruvate was assayed fluorometrically in a Farrand Optical model A-4 fluorometer by the method of Lowry and Passonneau (1972). Both pyruvate and arginine phosphate levels were determined immediately after neutralization to eliminate sample loss.

Aspartate, succinate, arginine, and malate were determined spectrophotometrically according to the method of Williamson and Corkey (1969), Williamson (1974), Grieshaber and Gäde (1976), and Williamson and Corkey (1969), respectively. Octopine and lactate were assayed fluorometrically essentially as outlined by Graham and Ellington (1985).

Concentrations of the free amino acids alanine and glycine were determined by HPLC on a Dionex amino acid analyzer with a Pierce amino acid column and buffers (Pierce Chemical Company). Alanopine and strombine concentrations were also determined by HPLC methods (Fiore *et al.*, 1984).

RESULTS

Enzyme activities

Freshly prepared extracts of the radula protractor muscles of *Busycon contrarium* displayed high activities of all four pyruvate reductases (Table I). Octopine dehydrogenase (ODH) had the highest activity of the enzymes assayed. Alanopine dehydrogenase (ADH) displayed somewhat lower activity followed by strombine (SDH) and D-lactate (LDH) dehydrogenases (Table I).

Contractile activity

Contractile activity under nearly anoxic conditions was maintained within 98% of initial values for the first 5 min of electrical stimulation (Fig. 1). There was a general trend towards a decline in force thereafter. This pattern was evident in all muscle preparations tested.

Metabolite levels in the tissue and the medium

Arginine phosphate levels declined at the onset of muscular activity, reached a minimum after 10 min, and remained relatively constant thereafter (Table II). Free arginine levels increased in the first 2.5 min of contractile activity, then fell to near control levels at the end of the experiment (Table II). Within 10 min, alanine levels were three times greater than levels measured initially, but returned to near control levels after 15 min (Fig. 2). Glycine levels did not change significantly during the 15

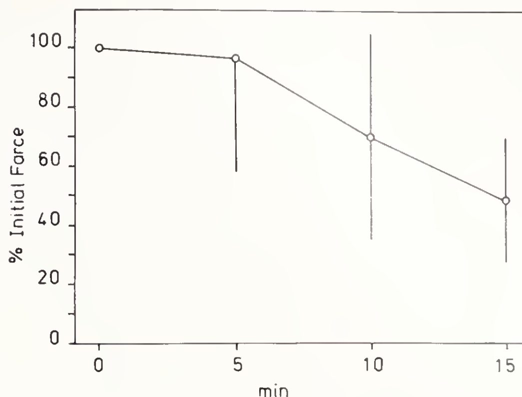

FIGURE 1. Changes in contractile force for electrically stimulated isotonic contractions of isolated radula protractor muscles from *Busycon contrarium versus* time. Units are percent change of initial force. Points represent means ± 1 SD, n = 5.

min of contractile activity (Fig. 2). Aspartate levels in the muscle declined during contractile activity, with the bulk of the change taking place in the first 5 min (Fig. 2). Succinate and malate levels in muscles stimulated for 15 min were less than 0.5 μmoles·g wet wt^{-1}.

After 2.5 min of stimulation, pyruvate levels in the muscle increased dramatically (Fig. 3). Strombine was not a major glycolytic end product, as this compound accumulated to levels approaching only 1 μmole·g wet wt^{-1} (Fig. 3). In contrast, octopine accumulated linearly during the experiment (Fig. 3, Table II). The sum of octopine, free arginine, and arginine phosphate levels was constant (Table II), indicating no net change in the total arginine pool in the tissue. Alanopine showed the highest accumulation of all the opines, but formation did not begin immediately as this compound was not detectable in the first 2.5 min of stimulation (Fig. 3). D-lactate was the predominant end product formed in the muscle, exhibiting a dramatic increase during the later periods of contraction (Fig. 3).

Octopine, alanopine, and strombine were not released into the medium by the muscle preparations. In contrast, a significant amount of D-lactate was found in the

TABLE II

Total arginine pool (arginine phosphate, arginine, octopine, and total arginine) for neutralized perchloric-acid extracts of isotonically contracting radula protractor muscles isolated from Busycon contrarium

Time (min)	Metabolite			
	Free arginine	Arginine phosphate	Octopine	Total
0	2.37 ± 2.41	10.69 ± 3.77	0.46 ± .29	13.47 ± 4.39
2.5	4.44 ± 1.17	7.35 ± 2.83	1.11 ± .85	12.55 ± 3.34
5	3.75 ± 1.04	6.98 ± 1.50	1.71 ± .38	12.44 ± 1.18
10	2.71 ± .83	4.93 ± 2.75	3.47 ± 1.64	10.76 ± 2.84
15	2.44 ± .48	6.98 ± 2.64	4.72 ± 1.73	14.14 ± 1.26

Levels are expressed in μmoles·g wet wt^{-1}. Data represent means ± 1 SD, n = 5.

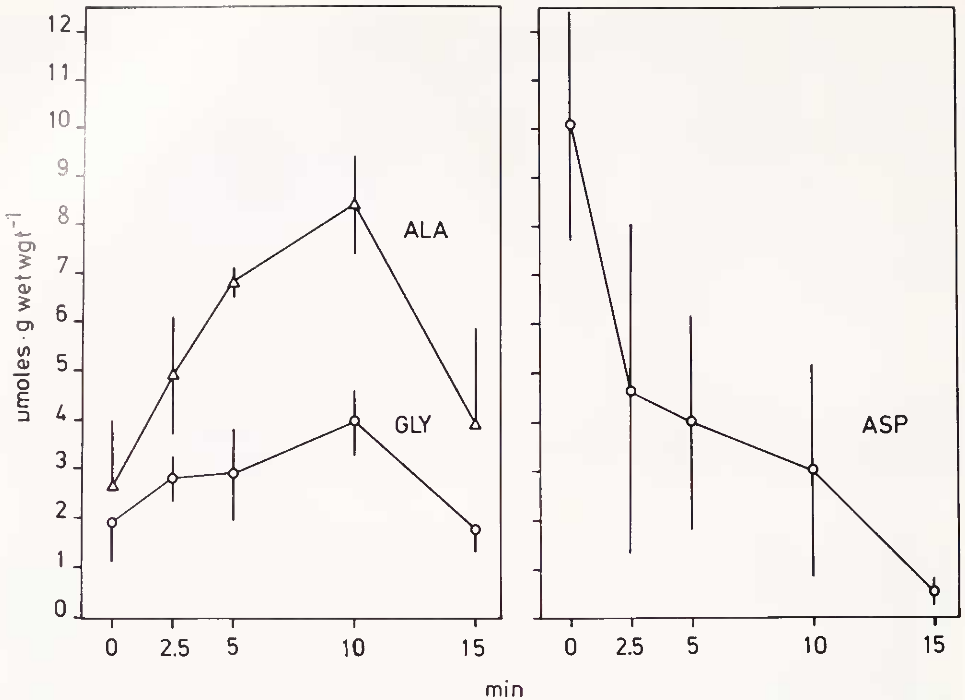

FIGURE 2. Changes in levels of the amino acids alanine (ALA), glycine (GLY), and aspartate (ASP) as determined by HPLC of neutralized perchloric-acid extracts of electrically stimulated radula protractor muscles from *Busycon contrarium* over time. Units are μmoles·g wet wt^{-1}. Points represent means ± 1 SD, n = 5.

medium (Fig. 4). In fact, during the last 5 min, more D-lactate was released into the medium than accumulated in the muscle (Fig. 4).

DISCUSSION

The high activities of all four pyruvate reductases in the radula protractor muscle of *B. contrarium* are similar to those observed in the radula retractor muscles of this species (Ellington, 1982) and in other gastropod muscles (Baldwin and England, 1982; Livingstone *et al.*, 1983). The highest activity, exhibited by octopine dehydrogenase, was comparable to activities observed in cephalopod molluscs (Baldwin and England, 1980). The simultaneous accumulation of D-lactate, alanopine, octopine, and to a lesser extent strombine, which occurs in the radula protractor system during muscular activity, indicates that all four of these reductases operate under functional anoxia. The accumulation of these end products is temporally correlated with changes in pyruvate levels. Pyruvate levels increased dramatically during the time course of muscle contraction.

Pyruvate reductases are thought to be equilibrium enzymes (de Zwaan and Dando, 1984; Gäde and Grieshaber, 1986) and are thus regulated by changes in the concentrations of substrates and products. Pyruvate is the common substrate for all four reductases. The observed elevations in pyruvate levels in *B. contrarium* radula

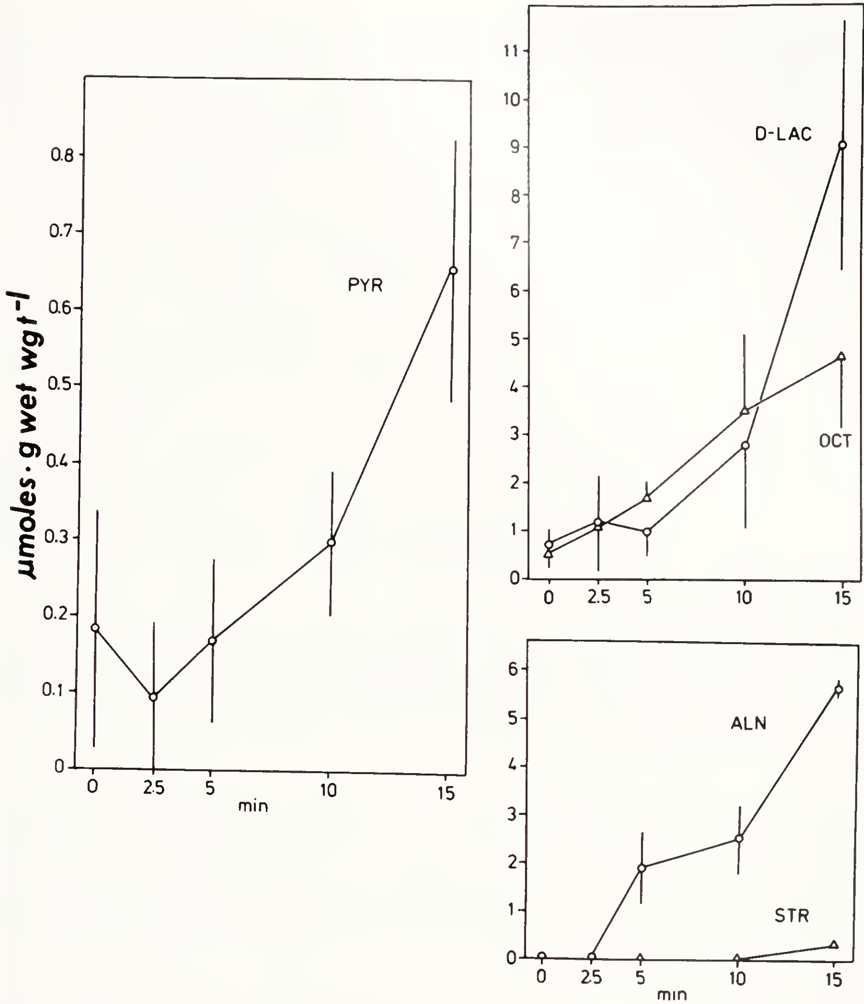

FIGURE 3. Pyruvate (PYR), D-lactate (D-LAC), octopine (OCT, alanopine (ALN), and strombine (STR) levels in electrically stimulated radula protractor muscles from *Busycon contrarium* over time, as determined by HPLC and fluorometry. Units are μmoles · g wet wt^{-1}. Points represent means ± 1 SD, n = 5.

protractor would undoubtedly enhance all pyruvate reductase activities. In the case of opine dehydrogenases, the concentrations of amino acid co-substrates are also important. In the radula protractor muscle, we found that alanine, glycine, and free arginine levels were in the 2–8 μmole·g^{-1} range. Finally, pyruvate reductases are influenced by accumulation of their respective products. Opine dehydrogenases seem to be particularly sensitive to product inhibition (Gäde and Grieshaber, 1986).

D-Lactate was the dominant glycolytic end product even though the maximal LDH activity measured in crude tissue extracts was the lowest. Isolated radula muscle of *B. contrarium* displayed the highest levels of D-lactate accumulation yet observed during functional anoxia in molluscs. Meinardus and Gäde (1981) observed a rela-

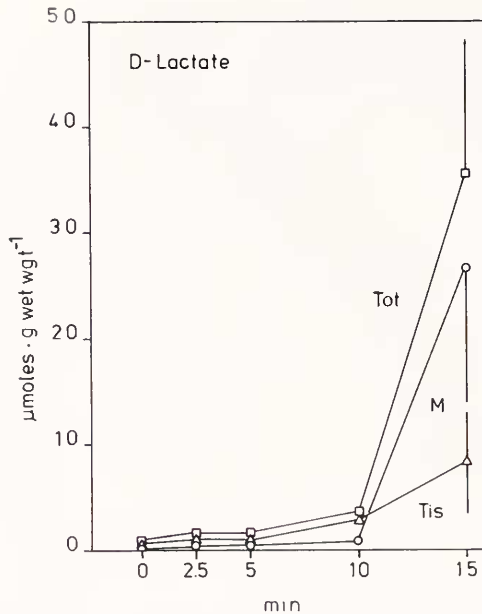

FIGURE 4. Distribution of lactate in the incubation media (M) and tissue (Tis) as well as the total (To) lactate produced during electrically induced isotonic contractions of the radula protractor muscles from the whelk *Busycon contrarium* as determined by fluorometric analysis of neutralized perchloric-acid extracts of each over time. Units are μmole · g wet wt^{-1}. Points represent means ± 1 SD, n = 5. Where error bars are absent, the error bar was less than the size of the symbol used to mark points.

tively modest accumulation of D-lactate in electrically stimulated preparations of the foot muscle of the cockle *Cardium edule.* The preferential production of D-lactate *versus* the opines in *B. contrarium* radula protractor muscle may be related to a higher binding capacity for pyruvate. The apparent Kms for pyruvate of molluscan LDHs are considerably lower than corresponding Kms of ODHs and somewhat lower than pyruvate Kms for strombine and alanopine dehydrogenases (Gäde and Grieshaber, 1986).

Isolated radula muscle preparations of *B. contrarium* did not release octopine, alanopine, or strombine into the medium. In addition, the total arginine pool (free arginine, arginine phosphate, and octopine) remained constant during contractile activity. The decline in aspartate levels during anoxia probably reflects transdeamination to alanine because, at the end of 15 min, the alanopine-alanine pool size was roughly equivalent to the decrease in aspartate plus initial alanine levels. This conservation of nitrogen in both the arginine (arginine, arginine phosphate, octopine) and alanine (alanine, alanopine, aspartate difference) pools is consistent with the observed absence of transport of opine end products out of the muscle.

In contrast to that of the opines, release of D-lactate from the muscle was very large. In fact, D-lactate export was greater than D-lactate accumulation during the 10–15-min period of observation. D-lactate release could have at least two major functional advantages. First, removal of the product of this reaction would change the mass action ratio ([lactate]/[pyruvate]) in favor of more product formation. Second, if lactate were transported out in a symport system with a proton (H^+) or in an antiport system (OH^-), this process would help the muscle cells regulate intracellular pH (pH_i).

The total D-lactate produced during the 15 min of contractile activity approached 50 μmoles·g wet wt^{-1}. On the basis of established proton stoichiometries of glycolysis (Pörtner *et al.*, 1984)—*i.e.*, one mole of protons (H^+) per mole lactate (or opine) produced—lactate and also opine production clearly impose a significant acid load on the muscle. Buffering capacities of whelk radular and ventricular muscle, as determined by the homogenate titration method (Castellini and Somero, 1981), ranged from 30.7 to 39.5 Slykes·g wet wt^{-1} (Eberlee and Storey, 1985; Graham and Ellington, 1985). However, the buffering capacity of *B. contrarium* ventricles, as determined by imposing an acid load (Ellington, 1985) and measuring pH_i by phosphorus nuclear magnetic resonance (^{31}P-NMR) spectroscopy, yielded a value approaching 24 Slykes·g wet wt^{-1} (Ellington, unpub. obs.). Regardless of the exact position of the buffering capacity, the acid load imposed on the muscle clearly could not be offset by purely passive means such as buffering.

Lactate transport has been studied extensively in erythrocytes. Three mechanisms of lactate transport have been identified: (1) non-ionic diffusion, (2) classical anion transport, and (3) monocarboxylate carrier (H^+ symport or OH^- antiport) mediated transport. Deuticke *et al.* (1982) reported at least three parallel pathways of lactate transport in erythrocytes. Lactate transport in erythrocytes has become the paradigm on which mitochondrial (Palmieri *et al.*, 1971), hepatocyte (Fafournoux *et al.*, 1985), and whole-muscle (Mainwood and Worsley-Brown, 1975; Seo, 1984) lactate transport have been modeled. The efflux of lactate from the radula protractor muscle of *B. contrarium* is probably caused by one or several of these mechanisms. Because the pKa of lactic acid is 3.86, and the intracellular pH of molluscan tissues under a variety of conditions ranges from 7.1 to 6.6 (Ellington, 1983a; Graham and Ellington, 1985), almost all the acid would dissociate into anions; therefore, the rates of non-ionic diffusion should be low. A lactate:H^+ symport (or lactate:OH^- antiport), if present in the radula protractor muscle of *B. contrarium*, could be of critical importance in regulating pH_i, especially during periods of elevated glycolytic rates.

In contrast to lactate, the opines are not released into the medium by the radula protractor muscle of *B. contrarium*. In fact, there is no *direct* evidence for release of opines from any molluscan tissue, although the increase in hemolymph levels during contractile activity in cephalopods (Storey and Storey, 1979; Gäde, 1980) and the decline in the total arginine pool in the mantle muscle during swimming in *S. officinalis* (Storey and Storey, 1979) and *Loligo vulgaris* (Grieshaber and Gäde, 1976) suggest indirectly that octopine is released in these cases. No other study of contractile activity in molluscs has revealed a decline in the total arginine pool or release of opines from the muscle (de Zwaan and Dando, 1984).

Why is lactate readily exported from molluscan muscle cells while opines appear to be retained? Specific transporters for amino acids are present in a wide range of cell types (Preston and Stevens, 1982); thus there is no fundamental impediment with respect to transport of these compounds. Opine formation results in no net increase in the number of osmotically active particles because the amino acid condenses with pyruvate derived from a large polymer (glycogen). In contrast, lactate formation results in an increase in osmotically active particles, as glycogen is broken down into smaller fragments. The lack of disturbance of internal osmolarity has been used as a potential functional explanation for the use of opine dehydrogenases rather than LDH in certain molluscan muscles (Zandee *et al.*, 1980; Fields, 1983). A logical derivative of the argument is that it would be disadvantageous to transport out opine end products. However, accumulated end products represent only a small fraction (<5%) of the pool of osmotically active substances. Thus, removal of end products, or lack thereof, for the sole purpose of cell volume regulation seems unlikely. We favor the

possibility that the lack of export of opines is related to the lack of a mechanism for coupling this movement with regulation of pH_i. In fact, since octopine, alanopine, and strombine have both positive and negative charges at prevailing pH_i conditions, a transport mechanism that could couple H^+ (symport) or OH^- (antiport) movement with opine export would be difficult to envision.

The absence of significant amounts of succinate or malate accumulation makes the fermentation of aspartate during functional anoxia unlikely. Although the carbon skeleton may be unaccounted for, nitrogen is balanced through alanine formation. Most probably, the amino group of aspartate is transaminated ultimately to pyruvate to yield alanine, with the carbon skeleton of aspartate entering the Krebs cycle as malate or oxaloacetate. This hypothesis entails the assumption that there is enough oxygen available to the cells to sustain a significant level of aerobic metabolism, at least during the early phases of contractile activity. The myoglobin content of radula muscle is high (Ball and Meyerhof, 1940; Fange and Mattisson, 1958), and molluscan myoglobins typically have low P_{50} values (Read, 1966). Glycolytic rates, as evidenced by end product accumulation, are low in the early phases of contractile activity, suggesting that energy production is largely aerobic during this period. Presumably, the oxygen used in this period could be derived from an internal oxygen store such as myoglobin. Contractile force decreases in the later portions of the time course while pyruvate concentrations increase, suggesting that there is a transition from aerobic to anaerobic processes. Thus, aspartate may be an aerobic substrate during the early phases of muscle contraction.

To sum up, our studies have shown that during contractile activity in the radula protractor muscle of *B. contrarium,* high glycolytic rates prevail with pyruvate being shunted through all the major pyruvate reductases. Strombine, octopine, alanopine, and D-lactate accumulated in order of increasing levels. D-lactate was the major end product although D-lactate dehydrogenase displayed the lowest *in vitro* activity of all pyruvate reductases. D-Lactate, but none of the opines, was released from the muscle into the incubation medium, with rates exceeding 3 μmoles $\cdot$ min^{-1} $\cdot$ g wet wt^{-1}. The removal of lactate from the muscle enhances the mass action ratio in favor of lactate formation, thereby increasing carbon flux through this enzyme. The removal of lactate from muscle cells during contractile activity may also help to regulate pH_i through a H^+ symport or OH^- antiport system. This potential role of lactate transport in the regulation of pH_i is currently the subject of intensive investigation in this laboratory.

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